Journal Pre-proofs


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PII: S0963-9969(23)00216-8
DOI: https://doi.org/10.1016/j.foodres.2023.112671
Reference: FRIN 112671

To appear in: *Food Research International*

Received Date: 12 November 2022
Revised Date: 13 January 2023
Accepted Date: 6 March 2023

Please cite this article as: Gonçalves, D.A., Alves, V.D., Teixeira, J.A., Nobre, C., Development of a functional prebiotic strawberry preparation by in situ enzymatic conversion of sucrose into fructo-oligosaccharides, *Food Research International* (2023), doi: https://doi.org/10.1016/j.foodres.2023.112671

# Development of a functional prebiotic strawberry preparation by *in situ* enzymatic conversion of sucrose into fructo-oligosaccharides

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## *Abstract*

Food industry has been pressed to develop products with reduced sugar and low caloric value, while maintaining unchanged their rheological and physicochemical properties. The development of a strawberry preparation for the dairy industry, with prebiotic functionality, was herein investigated by *in situ* conversion of its intrinsic sucrose content into prebiotic fructo-oligosaccharides (FOS). Two commercial enzymatic complexes, Viscozyme® L and Pectinex® Ultra SP-L, were evaluated for the synthesis of FOS. Operational parameters such as temperature, pH, and enzyme:substrate ratio (E:S) were optimized to maximize FOS yield. The rheological and physicochemical properties of the obtained strawberry preparation were evaluated. For functional analysis, the resistance of FOS to the harsh conditions of the gastro-intestinal digestion was evaluated by applying the standardized INFOGEST static protocol. At optimal conditions (60 ℃, pH 5.0), Pectinex® produced 265 ± 3 $g \cdot L^{-1}$ FOS, yielding 0.57 ± 0.01 $g_{FOS} \cdot g_{in.GF}^{-1}$ after 7hours reaction (E:S: 1:40); and Viscozyme® produced 295 ± 1 $g \cdot L^{-1}$ FOS, yielding 0.66 ± 0.00 $g_{FOS} \cdot g_{in.GF}^{-1}$ after 5 hours (E:S: 1:30). The obtained strawberry preparations contained more than 50 % (w/w) prebiotic FOS incorporated (DP 3−5), with 80 % reduction of its sucrose content. The caloric value was therefore reduced by 26−31 %. FOS showed resistance to gastrointestinal digestion being only slightly hydrolysed (< 10%). Fructo-furanosylnystose was not digested at any phase of the digestion. Although the physicochemical properties of the prebiotic preparations were different from the original one, parameters such as the lower *ºBrix*, water activity, consistency and viscosity, and its different color, may be easily adjusted. Results indicate that *in situ* synthesis strategies are efficient alternatives in the manufacture of reduced sugar and low-caloric food products with prebiotic potential.

***Keywords*:** fructo-oligosaccharides; *in situ* enzymatic synthesis; fructosyltransferase; strawberry preparation; functional food; food digestion

## 1. INTRODUCTION

Fruit preparations are intermediate products used in dairy, bakery, and confectionary food. They are excellent sources of vitamins and nutrients. According to Precedence Research Report (2022), the market size of global fruit-based preparations for dairy was valued at $5.96 billion in 2022 and it is expected to increase at a compound annual growth rate (CAGR) of 6.1 %, hitting $9.48 billion at the end of 2030 (Precedence Research, 2022). Of the flavors being commercialized, strawberry had the largest market size, accounting for 33 % of the total flavored-yogurt market share in 2019. And its market is expected to grow more than 7.6 % CAGR between 2020 and 2027 (Grand View Research, 2019).

Still, even preparations and juices made with 100 % fruit and without added sugar, may incorporate a high amount of intrinsic caloric sugars. Moreover, sugars and syrups added during the processing of food products account for an average of 270 calories ingested per day (Makarem et al., 2018). This excessive sugar consumption has been associated with an increased risk of diseases, such as cancer and obesity, as well as with a negative impact on mental health and cognitive function (Jacques et al., 2019). For this reason, the World Health Organization (WHO) advises that the intake of sugar should be reduced to less than 10 % of the total energy intake, for both adults and children, and encourages a further reduction to below 25 g per day (< 5 %) (World Health Organization, 2013).

In this context, several strategies have been implemented in various countries with the aim of reducing sugar consumption and empowering healthier choices. These include tax implementation to raise the price of sugary food products; restrict the marketing and advertising of unhealthy products; promote healthier options; ensure clear nutritional labeling; and restrict the access of unhealthy products in particular locations (*e.g.*, schools) (Muth et al., 2019). Also, a gradual and progressive sugar reduction appears to be one of the most promising solutions, along with product reformulation (Jawaldeh et al., 2018). Nonetheless, due to a lack of supervision, policies are not always adopted and followed, and sugar consumption remains excessive. Worldwide, around 19 % of the adult population (≥ 19 year-old) consumes more sugar than recommended. But even more worrisome are the levels among adolescents (13−18 year-old) and children (≤ 12 year-old) which reach the levels of 24 % and 12 %, respectively, with a rising trend (Walton et al., 2021).

The consciousness that health is intrinsically related to the food we chose to ingest has been growing. Thus, consumers are increasingly seeking food not only of good nutritional composition but also with health-promoting properties (Gonçalves et al., 2022). This has led to an increase of approximately 10 % in the functional food market in recent years, opening space for new differentiated functional products, such as food with prebiotic functionality (Birch and Bonwick, 2019). Prebiotics are food ingredients known to improve the composition of gut microbiota, which in turn improves health by preventing the development of diet-related disorders (Florowska et al., 2016). Additionally, prebiotics may alter and enhance the physicochemical and sensorial characteristics of food, being excellent ingredients for the development of new products (Nobre et al., 2015). Due to their appealing properties, the food industry has been incorporating prebiotics in a variety of products, as means of sugar or fat substitute (Farias et al., 2019; Varzakas et al., 2018). The most common strategy used has been the direct incorporation of the prebiotic into the product, either by fortification or *prior* to processing (Gonçalves et al., 2022). Much less reported is the direct synthesis of prebiotics in the product itself. Nonetheless, few authors have successfully applied the *in situ* approaches to reduce the caloric content of fruit juices (Baruah et al., 2017; Johansson et al., 2016; Nguyen et al., 2015). Advantageously, the *in situ* synthesis of prebiotics not only adds functionality to the food as simultaneously reduces its intrinsic sugar. Nevertheless, its impact on the physicochemical, texture, and stability of the final products must be considered.

In this view, this work aims to reduce the sugar content of a commercialized strawberry preparation for the dairy industry and at the same time develop a functional product by *in situ* enzymatic conversion of its sucrose into prebiotic fructo-oligosaccharides (FOS). Fruit preparations contain fruit (usually > 35 %) and can include other components such as essences, flavors, and coloring agents (Kurz et al., 2008). Aside from the natural sugars found in fruits, a large variety of sugars, but mainly sucrose, are often added during the processing or manufacturing of this type of product, increasing dramatically its caloric value (Monteiro-Alfredo et al., 2021). This high content of sucrose may although be an excellent substrate for the enzymatic synthesis of prebiotic FOS. FOS are fructose oligomers enzymatically synthesized through the transfructosylation of sucrose by enzymes showing fructosyltransferase activity (Nobre et al., 2022). The potential of two commercial enzyme preparations from *Aspergillus* sp., Viscozyme® L and Pectinex® Ultra SP-L, for the synthesis of FOS will be evaluated. A commercial strawberry preparation will be used as raw material for the development of a novel functional product with low-sugar and caloric content. The optimal conditions for the transfructosylation reaction, including temperature, pH, and enzyme concentration will be investigated. As well as the rheological and physico-chemical properties of the obtained enzymatic-treated samples. For evaluation of the stability of the prebiotic samples under the harsh conditions of the

gastrointestinal systems, samples will be digested following the harmonized static protocol from INFOGEST (Brodkorb et al., 2019).

## 2. MATERIALS AND METHODS

### 2.1. Materials

Pectinex® Ultra SP-L (≥ 3,800 U·mL$^{-1}$) and Viscozyme® L (≥ 100 FBGU·g$^{-1}$) were purchased from Sigma-Aldrich (Germany). The strawberry preparation (REF. INV59512 02) was kindly provided by Frulact SA (Maia, Portugal). FOS standards (1-kestose ($GF_2$), nystose ($GF_3$), and $1^F$-fructofuranosylnystose ($GF_4$)) were acquired from FUJIFILM Wako Chemicals (Japan). Sucrose, fructose, and glucose standards were purchased from Riedel-de Haën (Germany), Panreac (Germany), and Scharlau (Spain), respectively. Porcine pepsin, pancreatin, bile extract, pefabloc SC, $(NH_4)_2{\cdot}CO_3$, $NaHCO_3$, HCl (37 % (w/v)) were purchased from Sigma-Aldrich (St. Louis, MO). $MgCl_2{\cdot}(H_2O)_6$ was purchased from Merck (Germany). NaCl, KCl, NaOH, $KH_2PO_4$ and $CaCl_2{\cdot}(H_2O)_2$, were obtained from Panreac (Spain).

### 2.2. Methods

#### *2.2.1. Enzyme activity assays*

Transfructosylation and hydrolysis activities of the Pectinex® Ultra SP-L and Viscozyme® L were determined by the measurement of the glucose and fructose released from sucrose, following the method described by Vega and Zúniga-Hansen (2011) with a few modifications. The reaction mixture consisted of 1 mL of the enzyme preparation and 4.9 mL of a 45 % (w/v) sucrose solution in 100 mM sodium acetate buffer. The mixture was incubated for 30 min and stirred at 150 rpm. The reaction was stopped by heat-inactivating the enzyme in boiling water for 10 min.

One unit of the transfructosylation activity was defined as the amount of enzyme required to transfer 1 µmol of fructose per minute. The transferred fructose ($F_T$) in the reaction medium was calculated using **Eq. 1**, where F and G are the concentrations of fructose and glucose (µg/mL) in the mixture, respectively (Chen & Liu, 1996). Both fructose and glucose concentrations were determined by High Performance Liquid Chromatography (HPLC) (see **Section 2.2.5**). As fructose is exclusively produced from sucrose hydrolysis, the hydrolytic activity was defined as the amount of enzyme required to release 1 µmol of fructose per minute.

$$[F_T] = [G] - [F] \quad \textbf{(1)}$$

#### *2.2.1.1. Effect of the reaction conditions on the enzymes' activity and FOS synthesis*

By individually changing the parameters of the procedure outlined in **Section 2.2.1**, experiments were conducted to evaluate how the temperature and pH levels could affect the enzymes' activity, and consequently, the FOS synthesis. A volume of 0.3 mL of enzyme preparation was added to 14.7 mL of sucrose solution (45 % (w/v)) in sodium acetate buffer (100 mM). The effect of temperature was studied by keeping the reaction at 50, 55 and 60 °C, while the pH was kept at 4.0. The effect of the pH was tested by keeping the reaction at pH 4.0, 4.5, 5.0, and 5.5, with constant temperature held at 50 ℃. After 1-hour incubation, the reaction was stopped by heat-inactivating the enzyme in boiling water for 10 min. Samples were collected for carbohydrates quantification.

### *2.2.2. Optimization of the enzyme concentration for FOS synthesis*

Under the optimal reaction conditions, previously determined in **Section 2.2.1.1**, the effect of enzyme concentration on the synthesis of FOS was determined. Three enzyme:substrate ratios (E:S, v/v) of 1:50, 1:40, and 1:30 were evaluated. A sucrose solution of 45 % (w/v) was used as substrate in the experiments. The reaction was conducted for 8 h and stirred at 150 rpm. At 1-hour intervals, aliquots of 1 mL were withdrawn from the reaction mixture and boiled in water for 10 min to heat-inactivating the enzyme. Samples were collected for carbohydrates quantification.

The conditions maximizing FOS synthesis were used for the *in situ* conversion of sucrose in the strawberry preparations.

### *2.2.3. In vitro digestion of the prebiotic strawberry preparations*

*In vitro* digestion was performed following the INFOGEST 2.0 protocol. The enzymatic-treated strawberry sample in study was exposed to conditions simulating the oral, gastric, and intestinal phases. Electrolyte solutions, *i.e.*, simulated salivary fluid (SSF), gastric fluid (SGF), and intestinal fluid (SIF) were prepared following the protocol (Brodkorb et al., 2019). To avoid precipitation, the $CaCl_2 \cdot (H_2O)_2$ was not added to the stock electrolyte solutions. Instead, a 0.3 M $CaCl_2$ solution was prepared.

Briefly, the oral phase was simulated by mixing 4 mL of SSF, 25 µL of 0.3 M $CaCl_2$ (to obtain 0.15 mM in the fluid), and 975 µL of ultra-pure water to 5 mL of the strawberry preparation. The sample was incubated at 37 °C for 2 min. Note that alpha-amylase solution was not used, being substituted by ultra-pure water. Following, to simulate the gastric phase, the oral bolus was mixed

with 8 mL of SGF, 5 µL of $CaCl_2$ 0.3 M (to obtain 0.15 mM in the fluid), porcine pepsin solution (to obtain 2000 $U \cdot mL^{-1}$ in the digestion sample), 1 M HCl to adjust pH to 3.0 and purified water (to attain a final volume of 20 mL). The samples were incubated at 37 ℃, in a shaking bath for 2 h. The intestinal phase was simulated by adding SIF solution, $CaCl_2 \cdot (H2O)_2$ (to obtain 0.6 mM in the fluid), pancreatin suspension in SIF (to achieve a trypsin activity of 100 $U \cdot mL^{-1}$ in the final sample), bile solution in SIF (to obtain a final concentration of 10 mM in the sample), 1 M NaOH (volume necessary to adjust the pH to 7.0) and purified water (volume needed to dilute the mixture to a final volume of 40 mL). The samples were incubated in a shaking bath for 2 h at 37 °C. All electrolyte solutions were pre-warm in a 37 °C bath *prior* to use in the digestion procedures.

Aliquots were collected after each phase of the *in vitro* digestion for carbohydrates quantification. The gastric phase reaction was stopped by raising the pH to 7.0 with 1 M $NaHCO_3$.

### *2.2.4. Characterization of the strawberry preparations*

The pH of the enzymatic treated preparations was measured using a pH meter HI-2210 (Hanna Instruments). For accurate results, the instrument was *prior* calibrated using two known pH buffer solutions (4.0 and 7.0). The water activity (*aw*) was analyzed using the water activity meter AquaLab 4TE Dew Point (METER Group Inc, WA, USA) after an equilibrium at 25 °C. The total soluble solids (TSS) were determined by a portable refractometer HI-96801 (Hanna Instruments) by measuring the refractive index of the samples . The results were expressed in °*Brix* (g per 100 g), and distilled water was used as reference.

A CR-400 portable chroma meter (Konica Minolta, Japan) was used to perform the color measurements. *Prior* to the measurement, the instrument was calibrated against a white calibration plate (Pereira et al., 2021). Samples were placed in a reflectance glass recipient, and the measurements were performed at room temperature. The CIELAB coordinates $L^*$ (brightness–darkness), $a^*$ (redness–greenness), and $b^*$ (yellowness–blueness) were obtained directly from the instrument. Three additional parameters, including color differences ($\Delta E$**; Eq. 2**), chroma ($C^*$; **Eq. 3**), and hue angle ($h°$; **Eq. 4**) were calculated.

$$\Delta E = \sqrt{(\Delta a^{*2} + \Delta b^{*2} + \Delta L^{*2})} \quad \textbf{(2)}$$

$$C^* = \sqrt{(a^{*2} + b^{*2})} \quad \textbf{(3)}$$

$$h° = tan^{-1}(b^* \times a^*) \quad \textbf{(4)}$$

The difference between samples ($\Delta E$) were estimated according to the following scale: (i) not noticeable: 0–0.5; (ii) slightly noticeable: 0.5–1.5; (iii) noticeable: 1.5–3.0; (iv) detectable: 3.0–6.0; or (v) highly detectable (6.0–12.0) (Cserhalmi et al., 2006).

Rheological and viscoelastic measurements were performed at 25 ℃. The experiments were carried out in a TA Instruments HR-1 Rheometer equipped with a Peltier plate (TA Instruments, New Castle, DE), with a cone-plate (60 mm, 2° angle, truncation 64 μm). To acquire the respective flow curves, three rheological measurements were conducted: (i) the first with increasing shear rate (2.5–500 $s^{-1}$); (ii) the second with descending shear rate (500–2.5 $s^{-1}$); and (iii) the third with increasing shear rate (2.5–500 $s^{-1}$). Data from the last curve was fitted to the model of the power-law (**Eq. 5**), where $\sigma$ is the shear stress (Pa), $k$ is the consistency coefficient (Pa·$s^n$), $\gamma$ is the shear rate ($s^{-1}$), and $n$ is the flow behavior index (Vieira et al., 2021).

$$\Sigma = k \times \gamma^n \quad \textbf{(5)}$$

Viscoelastic properties of the strawberry samples were evaluated by performing a frequency sweep between 0.1 and 10 Hz at a constant strain of 1.0 %. The storage ($G'$, Pa) and loss ($G''$, Pa) moduli, and loss tangent (tan $\delta$, dimensionless) were analyzed to determine the prevailing elastic or viscous behavior of the strawberry samples. The complex viscosity ($\eta^*$), which is correlated with the perception of the thickness (He et al., 2016), was also evaluated. All data were exported directly from the equipment software (TRIOS 5.1.1).

### *2.2.5. Carbohydrate quantification*

The carbohydrates contained in the strawberry samples were analyzed using a HPLC system (Jasco) equipped with a refractive index detector and an autosampler. The separation was performed with an Asahipak NH2P-50 4E column (4.6 × 250 mm, 5 μm particle size) linked to an Asahipak NH2P-50G 4A pre-column (4.6 mm × 10 mm) from Shodex (Japan). Samples (20 μL) were eluted with a mixture of acetonitrile (HPLC Grade, Fisher Chemicals, USA) in Milli−Q water (70:30 v/v) and 0.04 % of ammonium hydroxide in water (HPLC Grade, Sigma-Aldrich, Germany). The elution was conducted at a flow-rate of 1 mL·$min^{-1}$. The Column temperature was maintained at 30 ℃ (Nobre, Gonçalves, et al., 2018). The chromatographic signal was recorded and integrated using the Star Chromatography Workstation software 6.3 (Varian, USA).

### *2.2.6. Statistical analysis*

All experiments were performed in triplicate. The average values with standard deviation are reported. Results were analyzed using one-way analysis of variance (ANOVA) followed by either Tukey's or Dunnett's multiple comparison tests at a 95 % confidence level. Differences were considered significant at $p$-values < 0.05. All data were analyzed using GraphPad Prism 9.4.1 software (GraphPad Software, USA).

## 3. RESULTS AND DISCUSSION

### 3.1. Optimization of the reaction conditions in a model sucrose solution

The enzymatic activity of the commercial enzyme mixtures Viscozyme® L and Pectinex® Ultra SP-L towards the synthesis of FOS was investigated. Both enzyme mixtures demonstrated hydrolytic and transfructosylation activity (**Table 1**). According to the data, transfructosylation was the primary activity of both enzymatic preparations, whereas the hydrolytic activity towards sucrose was secondary. Under the tested conditions, Viscozyme® L obtained the highest levels of transfructosylation and hydrolysis activity. Similar findings were reported by Vega-Paulino and Zúniga-Hansen (2012) for the same enzymatic preparations.

**Table 1.** Effect of the temperature and pH on the transfructosylation (TU) and hydrolytic (HU) activities of the commercial enzyme preparations Pectinex® Ultra SP-L and Viscozyme® L.

| Factor | Treatment level | Pectinex® Ultra SP-L | | Viscozyme® L | |
|---|---|---|---|---|---|
| | | TU | HU | TU | HU |
| **pH** | 4.0 | 1.68 ± 0.05a | 0.45 ± 0.00 | 2.62 ± 0.04ab | 0.68 ± 0.00 |
| **(T = 50 ℃)** | 4.5 | 1.74 ± 0.04a | 0.28 ± 0.01 | 2.62 ± 0.05ac | 0.47 ± 0.00 |
| | 5.0 | 1.75 ± 0.04a | 0.17 ± 0.00a | 2.71 ± 0.05bcd | 0.35 ± 0.01a |
| | 5.5 | 1.80 ± 0.10a | 0.17 ± 0.00a | 2.79 ± 0.07d | 0.36 ± 0.00a |
| **Temperature** | 50 | 1.68 ± 0.05 | 0.45 ± 0.00 | 2.62 ± 0.04 | 0.68 ± 0.00 |
| **(pH = 4.0)** | 55 | 1.84 ± 0.02 | 0.46 ± 0.01 | 2.83 ± 0.06 | 0.69 ± 0.03 |
| | 60 | 2.03 ± 0.06 | 0.58 ± 0.01 | 3.00 ± 0.08 | 0.81 ± 0.02 |

Analysis was performed in independent triplicates. Values are expressed in mean ± standard deviation. Values in the same column with a common letter indicate no statistical difference among conditions ($p \geq 0.05$).

Both temperature and pH influence the transfructosylation and hydrolytic activity of the Viscozyme® L mixture. Pectinex® Ultra SP-L had both activities influenced by the temperature. Nonetheless, the pH did not affect the transfructosylation activity of Pectinex® Ultra SP-L ($p \geq 0.05$) but affected its hydrolytic activity. In the tested range, the temperature had the most impact on the transferase activity. The obtained results suggest that increasing the temperature and the pH will also increase the transferase reaction rate. Herein, between 50 and 60 ℃, the transfructosylation activity increased around 1.21 and 1.15-fold for Pectinex® Ultra SP-L and Viscozyme® L, respectively. Meanwhile, with pH, an increase of approximately 1.07-fold in the transfructosylation between 4.0 and 5.5 was verified for both enzyme mixtures. According to the obtained data, higher levels of transfructosylation are obtained by increasing the temperature. The disadvantage is that the hydrolytic activity also increases. The pH plays an important role as it

can be used to suppress hydrolysis. By increasing the pH from 4.0 to 5.5, the hydrolytic activity was reduced approximately 1.20 and 0.78-fold for Viscozyme® L and Pectinex® Ultra SP-L, respectively.

The best temperature for transfructosylation of sucrose into FOS was achieved at 60 ℃ for both enzyme mixtures. Similar findings were reported in the literature. Lorenzoni et al (2014) determined that the range of temperature favoring the transferase activity of Viscozyme® L was between 58 °C and 66 ℃, for a pH 5.5 and 60 % (w/v) sucrose. Also, an optimum temperature of 60 ℃ was reported for a transferase from Pectinex® Ultra SP-L in two other studies using the same sucrose concentration (Ghazi et al., 2007; Nemukula et al., 2009).

The effect of pH on the enzyme's activity was investigated by varying pH values. At a pH between 4.5 and 5.5, no significant differences ($p \geq 0.05$) in the transfructosylation activity of Pectinex® Ultra SP-L were observed, whereas for Viscozyme® L there were no differences between pH 5.0 and 5.5. The hydrolytic activity differed significantly between pH 4.5 and 5.0 ($p < 0.05$) for both enzyme preparations, but not between 5.0 and 5.5 ($p \geq 0.05$). The maximal transfructosylation with the lowest hydrolytic activity was observed at pH 5.5. Despite the differences in the reaction conditions, the results obtained in this study are in accordance with some reported data. In a reaction performed at a temperature of 65 ℃ and 60 % (w/v) sucrose solution, Pectinex® Ultra SP-L showed an optimum pH between 5.5 and 6.5 (Tanriseven & Aslan, 2005). Likewise, Pectinex® Ultra SP-L has been found to be suitable for FOS synthesis at pH levels higher than 4.0, using a 50 % (w/v) sucrose solution and a temperature of 50 ℃ (Veljković et al., 2021). The hydrolytic activity of FTase enzymes has shown to prevail at pH levels around 4.0, resulting in lower FOS yields. Furthermore, a pH of 5.6 was shown to promote transfructosylation instead of hydrolytic activity (Nemukula et al., 2009).

Nonetheless, since the aim of this study was to apply the enzyme preparations in strawberry preparations with original pH of 4.0 ± 0.2, and as changes in the product properties must be kept at a minimum, pH values lower than 5.5 could represent the best choice. Since no significant differences ($p \geq 0.05$) were found in the transfructosylation activity of both enzymes between 5.0 and 5.5, a pH value of 5.0 was considered in further experiments.

Regarding the ratio between the transferase and hydrolytic activities, the predominance of the transfructosylation activity is clear. FTase from Pectinex® Ultra SP-L shows greater transferase activity as a higher ratio was determined (11.86 ± 0.01), as compared to that of Viscozyme® L (9.25 ± 0.14). A high transferase/hydrolytic ratio is required to promote sucrose conversion and avoid FOS hydrolysis (Cunha et al., 2019). At the defined optimal conditions (60 ℃, pH 5.0)

Viscozyme® L showed 3.63 ± 0.06 transferase activity, whereas Pectinex® Ultra SP-L showed 2.41 ± 0.05. Based on these results, the same conditions were considered in the following assays.

### 3.2. Effect of enzyme concentration on FOS synthesis in a model sucrose solution

The amount of enzyme applied in food has an influence on the velocity of the reaction and on the amount of prebiotic synthesized. High enzyme concentrations have been shown to accelerate the enzymatic reaction, yielding also maximal concentrations of FOS produced (Hang & Woodams, 1996; Khatun et al., 2020). Nevertheless, the amount of enzyme present in a batch setup is constant, so the number of enzyme active sites remains constant along the reaction, getting successively reutilized throughout the reaction time (Kashyap et al., 2015). Therefore, the maximal FOS synthesis always depends on the number of active sites available.

As the present reaction is substrate-limited (45 % (w/v) sucrose), some of the catalytic sites may not be utilized if the available enzyme exceeds the amount of substrate. Furthermore, adding more enzyme than required will not increase the reaction rate as it only depends on the enzyme activity itself. For this reason, the influence of the enzyme concentration on FOS synthesis was investigated. The goal was to maximize the overall amount of FOS while keeping the costs at a minimum. The price of a food product is directly affected by the process used in its manufacture. For this reason, a commitment between enzyme levels and faster reaction times is important to reduce the overall production cost.

As depicted in **Fig. 1A and B**, a high enzyme:subtrate ratio (1:30, v/v) results in higher catalytic rates and faster FOS synthesis. This outcome is more evident during the first 5 hours of reaction. After this point, the catalytic reaction either stabilizes or slows down. This occurs because all active sites are occupied or due to substrate limitation. For Viscozyme® L, an enzyme:substrate ratio of 1:30 (v/v) allowed the highest FOS synthesis in a shorter amount of time. This commercial mixture was able to synthesize 280 ± 1 $g \cdot L^{-1}$ of FOS after 5 hour-reaction. As for Pectinex® Ultra SP-L, 270 ± 1 $g \cdot L^{-1}$ of FOS were synthesized after 8 hours using an enzyme:substrate of 1:40 (v/v). Nonetheless, at the highest enzyme dosage evaluated, a maximum of 257 ± 2 $g \cdot L^{-1}$ of FOS were obtained after 5 hours. This result was not statistically different ($p \geq 0.05$) from that obtained for an enzyme:ratio of 1:40 (v/v) at the same reaction time.

Briefly, for further experiments, an enzyme:substrate ratio of 1:30 (v/v), for Viscozyme® L, and 1:40 (v/v), for Pectinex® Ultra SP-L were chosen. The experimental conditions previously optimized (see **Section 3.1**) were employed.

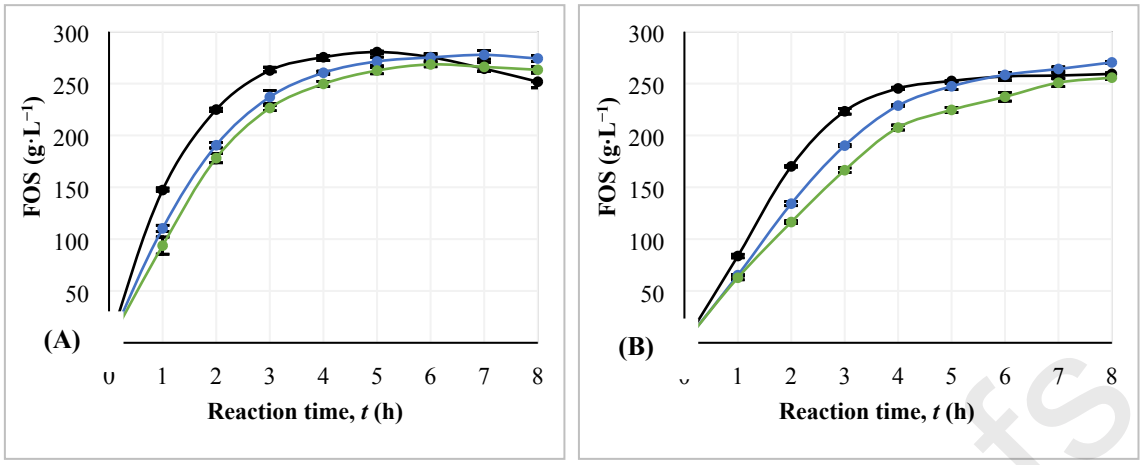


**Figure 1.** Impact of the concentration of the commercial enzyme Viscozyme® L (**A**) and Pectinex® Ultra SP-L (**B**) on FOS synthesis. Enzyme:substrate ratio (v/v) of 1:50 (green); 1:40 (blue); and 1:30 (black). Experimental conditions were 60 °C and pH 5.0. Data correspond to the mean ± standard deviation of independent triplicate assays.

### 3.3. FOS synthesis at optimal operational conditions on a model sucrose solution

Both commercial enzyme preparations showed to be capable of producing FOS in sucrose solutions. **Fig. 2A, and B** depict the enzyme reactions' progress under the operational conditions previously optimized for Viscozyme® L and Pectinex® Ultra SP-L (see **Section 3.1** and **3.2**). The process catalyzed by Viscozyme® L was faster with a maximum FOS production of 293 ± 1 $g \cdot L^{-1}$ after 5 hours reaction. Compared with the aforementioned concentration, Pectinex® Ultra SP-L generated substantially less FOS and took longer to achieve it. Maximum production of 265 ± 4 $g \cdot L^{-1}$ of FOS was attained after 7 hours reaction.

In the reaction catalyzed by Viscozyme® L, from 3 hours onwards, $GF_2$ started being hydrolyzed, while $GF_3$ and $GF_4$ were continuously produced (**Fig. 2A**). Most likely $GF_2$ was being used as a substrate for $GF_3$ and $GF_4$ production, as described by Jung et al. (1989). Microbial fructosyltransferases catalyze a series of disproportionation reactions in which a fructosyl moiety of a donor molecule, that can be GF or a newly formed FOS, is transferred to an acceptor molecule (Antosova & Polakovic, 2001). As a result, a mixture of FOS is generated, and glucose is released as a by-product. Ultimately, glucose accounted for 31 % of the total carbohydrates in the reaction medium, exceeding values of 100 $g \cdot L^{-1}$ from 2 hours onwards. Those high glucose levels have an inhibitory effect on the enzyme, impairing its transferase activity (Nobre, Alves Filho, et al., 2018). Alvarado-Huallanco and Maugeri-Filho (2010) reported that glucose not only inhibits sucrose transfructosylation but also $GF_2$ and $GF_3$.

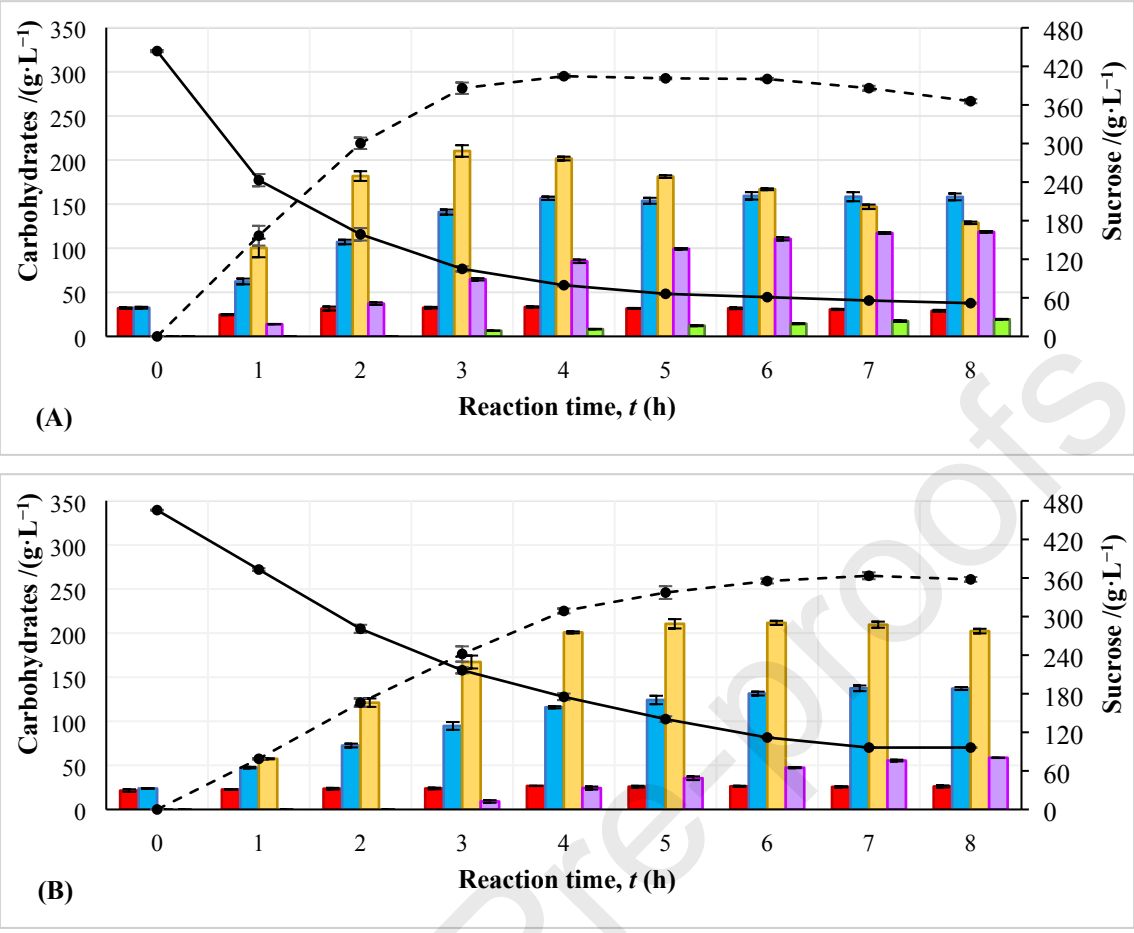


**Figure 2.** Time course of the FOS synthesis from sucrose catalyzed by Viscozyme® L (**A**), and Pectinex® Ultra SP-L (**B**). Experimental conditions were 60 °C and pH 5.0. Sucrose (—); Fructose (red); Glucose (blue); Total FOS (- - -); 1-Kestose, $GF_2$ (yellow); Nystose, $GF_3$, (lilac); and $1^F$-fructofuranosylnystose, $GF_4$ (green). Data correspond to the mean ± standard deviation of independent triplicate assays.

Pectinex® Ultra SP-L showed lower hydrolytic activity in the presence of high glucose concentrations as compared to Viscozyme® L. 1-Kestose was produced more slowly, reaching a maximum production between 5 and 6 h, and only afterward its concentration was slightly reduced (**Fig. 2B**). At the same time, $GF_3$ was progressively synthesized and $GF_4$ was barely detected (<1 $g \cdot L^{-1}$). The higher transferase/hydrolase ratio of this commercial preparation may explain the results achieved. Ghazi et al (2007) also verified that the rate of transfructosylation for Pectinex® Ultra SP-L was up to 20-fold higher than that of hydrolysis for sucrose concentrations above ≈340 g/L.

Viscozyme® L yielded 0.66 ± 0.00 $g_{FOS} \cdot g_{initial\ sucrose}^{-1}$, while Pectinex® Ultra SP-L 0.57 ± 0.01 $g_{FOS} \cdot g_{initial\ sucrose}^{-1}$. At optimal reaction points, the obtained reaction mixtures included 26−28 % glucose, 12−18 % of unreacted sucrose, and 5−6 % fructose. For microbial fructosyltransferases, due to the significant amounts of glucose released during the transfructosylation reaction, production yields between 0.55 and 0.60 $g_{FOS} \cdot g_{initial\ sucrose}^{-1}$ are normally expected (Nobre, Gonçalves, et al., 2018). Although Pectinex® Ultra SP-L had obtained a lower yield than

Viscozyme® L, values are in accordance with the expected range. Other authors obtained different yields for the same enzymatic preparations used herein, which may be related to the operational conditions applied. Lorenzoni et al (2014) only obtained an average yield of 0.55 $g_{FOS} \cdot g_{initial\ sucrose}^{-1}$ for Viscozyme® L for a pH 5.5 and 50 ℃, while Vega-Paulino and Zúniga-Hansen (2012) obtained 0.56 ± 0.12 for Viscozyme® L and 0.61 ± 0.03 $g_{FOS} \cdot g_{initial\ sucrose}^{-1}$ for Pectinex® Ultra SP-L at the same mentioned operational conditions.

In the present study, Pectinex® Ultra SP-L produced 210 ± 3 $g \cdot L^{-1}$ $GF_2$, and 55 ± 1 $g \cdot L^{-1}$ $GF_3$ using a pH of 5.0, a temperature of 60 ℃ and an of 1:40 (optimal conditions). On the other hand, Viscozyme® L synthesized 181 ± 2 $g \cdot L^{-1}$ $GF_2$, 99 ± 1 $g \cdot L^{-1}$ $GF_3$, and 12 ± 1 $g \cdot L^{-1}$ $GF_4$ at pH 5.0, 60 ℃ and with an enzyme:substrate ratio of 1:30 (v/v). Therefore, for *in situ* conversion of sucrose content in a commercial strawberry preparation the same conditions were selected for the experiments.

### 3.4. Application of the commercial enzymes in strawberry preparations

Chromatographic results have shown that the sugar composition of the studied strawberry preparation included 450 ± 5 $g \cdot L^{-1}$ sucrose, 28 ± 2 $g \cdot L^{-1}$ glucose, and 25 ± 2 $g \cdot L^{-1}$ fructose. The strawberry preparation was manufactured by a local food industry which commercializes the preparations for the dairy industry, mostly to be added to yogurts. From the information obtained from the supplier, 45 % (w/w) sucrose was added to the strawberry to manufacture the preparation. Therefore, most sucrose present in the preparation was added during the processing. In addition to sucrose, strawberries contain significant levels of glucose and fructose (Akšić et al., 2019). Thus, it was expected that in addition to the sucrose added during the production, some sugars naturally occurring in the fruit would likely represent part of the total sugar found in the final product formulation.

A high amount of sucrose must be present in the food to act as a substrate when applying the *in situ* synthesis of FOS, with enzymes exhibiting fructosyltransferase activity. Herein 45 % (w/w) of sucrose was determined in the fruit preparation. Some authors have claimed that to limit the hydrolytic activity of the enzymes, a sucrose concentration higher than 60 % (w/v) must be applied (Hernández et al., 2018). Nonetheless, it has been also reported that sucrose concentrations between 40 and 70 % (w/v) generate similar FOS yields (Vega-Paulino & Zúniga-Hansen, 2012). In the transfructosylation process, one sucrose molecule will act as a donor and another one as an acceptor (Antosova & Polakovic, 2001). As a result, the rate of FOS synthesis is highly influenced by the initial sucrose concentration.

In the present study, we aimed to transform marketed food into a healthier product. For this reason, the strawberry preparation with its original formulation was used, meaning no adjustment of the sucrose concentration. For the *in situ* enzymatic conversion of sucrose into FOS, the two commercial enzymatic mixtures were directly applied in the strawberry preparation. The operational conditions previously optimized with the sucrose model solution (450 $g \cdot L^{-1}$) were used (see **Section 3.3**). Briefly, a temperature of 60 ℃ and pH of 5.0 was applied and the reaction was stopped after 5 h reaction for Viscozyme® L, and 7 h for Pectinex® Ultra SP-L.

**Table 2** shows the carbohydrate content of the strawberry preparations after the enzymatic treatment. Sucrose concentration decreased from 450 ± 5 $g \cdot L^{-1}$ to average values of 83 ± 5 $g \cdot L^{-1}$ and 66 ± 2 $g \cdot L^{-1}$ after the treatments with Pectinex® Ultra SP-L and Viscozyme® L, respectively. It is therefore possible to infer that more than 80 % of the sucrose present in the strawberry preparation was used as the substrate for the synthesis of FOS. The fructose content remained nearly unchanged throughout the reaction, suggesting that the hydrolytic activity of the enzymes was low. Whilst glucose significantly rose after the enzymatic process, suggesting that the transfructosylation reaction was favored.

**Table 2.** Content of FOS and sugars in the final strawberry preparations after the enzymatic treatments with Pectinex® Ultra SP-L and Viscozyme® L.

| | | Sample | | |
|---|---|---|---|---|
| | | **Non-treated** | **Treated with Pectinex Ultra® SP-L** | **Treated with Viscozyme® L** |
| **Carbohydrate ($g \cdot L^{-1}$)** | **F** | 25 ± 2 | 26.4 ± 0.3 | 33 ± 1 |
| | **G** | 28 ± 2 | 137 ± 3 | 159 ± 1 |
| | **GF** | 450 ± 5 | 83 ± 5 | 66 ± 2 |
| | **$GF_2$** | | 189 ± 2 | 180 ± 4 |
| | **$GF_3$** | | 70 ± 2 | 102 ± 3 |
| | **$GF_4$** | | 6.0 ± 0.3 | 13 ± 1 |
| **Total FOS ($g \cdot L^{-1}$)** | | | 265 ± 3 | 295 ± 1 |
| **% FOS ($g_{FOS} \cdot g_{total\ carbohydrates}^{-1}$)** | | | 51.8 ± 0.4 | 53.2 ± 0.1 |
| **FOS Yield (% $g_{FOS} \cdot g_{initial\ sucrose}^{-1}$)** | | | 58.1 ± 0.6 | 66.4 ± 0.4 |
| **Converted Sucrose (%)** | | | 81.9 ± 1.1 | 85.0 ± 0.5 |

F— Fructose; G— Glucose; GF— Sucrose; $GF_2$— 1-Kestose; $GF_3$— Nystose; $GF_4$— $1^F$-Fructofuranosylnystose; FOS— Fructo-oligosaccharides.

At 5-hours reaction, Viscozyme® L synthesized significant levels of $GF_2$ (180 ± 4 $g \cdot L^{-1}$) and $GF_3$ (102 ± 3 $g \cdot L^{-1}$), as well as some amount of $GF_4$ (13 ± $g \cdot L^{-1}$). Similar results were obtained for Pectinex® Ultra SP-L that produced 189 ± 2 $g \cdot L^{-1}$ of $GF_2$, followed by 70 ± 2 $g \cdot L^{-1}$ of $GF_3$, and 6.0 ± 0.3 $g \cdot L^{-1}$ of $GF_4$ after a 7hour treatment (**Figure S1**). Results showed that the strawberry preparation was fortified with more than 50 % (w/w) of prebiotic oligosaccharides. More precisely, the enzymatic treatments resulted in a strawberry preparation containing approximately 25−30 g of FOS per 100 mL of food.

### 3.5. *In vitro* digestion of the prebiotic strawberry preparations

A standardized *in vitro* method, INFOGEST (Brodkorb et al., 2019), was used to investigate the digestion of the FOS found in the enzymatic-treated strawberry preparations. **Table 3** shows the hydrolysis degrees obtained by simulating the environment of three upper-digestion phases: (i) oral— mouth; (ii) gastric— stomach, and (iii) intestinal— small intestine. The percentage of each FOS in each phase was calculated by the difference between the amount of FOS at the beginning and at the end of the respective digestion phase (Nobre, Sousa, et al., 2018). Only results with a degree of hydrolysis higher than 2.0 % were considered significant ($p < 0.05$).

Results showed that only a minor percentage (< 0.5 %) of FOS from the enzymatic-treated strawberry preparations were hydrolyzed by the salivary fluid. 1-Kestose showed the lowest resistance to hydrolysis. A degradation of 2.8 % $GF_2$ in the gastric phase of the digestion was observed for Viscozyme® L, whilst 2.1 % was observed for Pectinex® Ultra SP-L treated sample. A more significant hydrolysis of $GF_2$ was noted in the intestinal phase. $GF_2$ was reduced in 7.1 % from its initial amount in the strawberry preparation treated with Pectinex® Ultra SP-L, and in 4.3 % with Viscozyme® L. Nystose showed more resistance to hydrolysis than $GF_2$. A reduction of 0.9 % of the $GF_3$ amount from Pectinex® Ultra SP-L treated sample and 1.3 % from Viscozyme® L was found at the end of the gastric phase. Furthermore, the $GF_3$ initial amount was reduced by 2.1 % and 1.7 %, respectively, at the end of the digestion. Fructo-furanosylnystose was not significantly digested in any digestion phase. Thus, $GF_4$ has more resistance to hydrolysis than the other two oligosaccharides.

**Table 3**. *In vitro* digestion of the strawberry preparations containing prebiotic FOS.

| Digestion phase (%) | Sample treated with Pectinex® Ultra SP-L | | | Sample treated with Viscozyme® L | | |
|---|---|---|---|---|---|---|
| | **$GF_2$** | **$GF_3$** | **$GF_4$** | **$GF_2$** | **$GF_3$** | **$GF_4$** |
| Mouth | 0.1 | 0.0 | 0.0a | 0.3 | 0.1 | 0.0 |
| Stomach | 2.1 | 0.9 | 0.0a | 2.8 | 1.3 | 0.1a |
| Small Intestine | 7.1 | 2.1 | 0.1 | 4.3 | 1.7 | 0.1a |

$GF_2$— 1-Kestose; $GF_3$— Nystose; $GF_4$— $1^F$-Fructofuranosylnystose. The values are expressed as rate of hydrolysis at different phases of the digestion. Values in the same column with a common letter indicate no statistical difference between phases ($p \geq 0.05$).

To consider the developed strawberry preparation as a potential prebiotic product, it is necessary to guarantee that a significant amount of the synthesized FOS reaches the large intestine. Prebiotic susceptibility to hydrolysis is largely affected by its chemical structure, as shown by the results. FOS are linear oligosaccharides with $\beta(1,2)$-linkages between its fructosyl units having a terminal glucose unit linked by an $\alpha(1,2)$-linkage. It is well established that there is no digestive enzyme

able to break the $\beta$(1,2)-glycosidic bounds (Sancho et al., 2017). However, both α-glycosidic and $\beta$(2,1) linkages are susceptible to the acidic conditions of the digestive system and may affect FOS hydrolysis resistance (Jackson et al., 2022; Nobre et al., 2019). Some studies have shown that FOS glycosidic linkages are highly sensitive to hydrolysis at low pH (Courtin et al., 2009) and their digestibility is more pronounced for the shorter chain FOS (Guimarães et al., 2020). Likewise, smaller fructans have shown more susceptibility for enzymatic hydrolysis in the small intestine, particularly 1-kestose (Nobre, Sousa et al. 2018; Nobre et al. 2019), contributing to an increased release of monosaccharides during digestion.

Most studies on carbohydrates digestion have been conducted using the standardized INFOGEST method. Nevertheless, current state-of-the-art has shown a few limitations of the protocol by the lack of small intestine mucosal carbohydrases, such as sucrase-isomaltase, which target sucrose-based carbohydrates (Hernandez-Hernandez et al., 2019). Despite the scarce studies, the high hydrolase resistance of FOS to mammalian digestive enzymes has been demonstrated. FOS were resistant to rat small intestinal mucosa homogenates and when ingested were scarcely hydrolyzed by the digestive enzymes of the rat (Oku et al., 1984). FOS showed also a low hydrolysis degree of 12.0 % when digested with rat small intestinal extract (Ferreira-Lazarte et al., 2017). *In vivo*, similar results have been found. When ingested by healthy volunteers, FOS were only 11% hydrolyzed in the small intestine.

Although in this work the INFOGEST digestion method has been applied, results were in good agreement with previous findings, since only less than 10 % of FOS were hydrolyzed during the digestion of the produced prebiotic strawberry preparations.

## 3.6. Characterization of the prebiotic strawberry preparations

### *3.6.1. Changes in the physico-chemical properties of the strawberry preparations*

One of the main goals for the development of the present *in situ* strategy was the caloric reduction of a commercial strawberry preparation, without significantly affecting its sweetness profile. **Table 4** shows both parameters before and after the enzymatic treatments. After the enzymatic treatment, the carbohydrate composition of the preparations significantly changed. Therefore, the caloric value of the preparations also decreased to 1358 ± 10 (Pectinex® Ultra SP-L) and 1453 ± 6 kCal·g$^{-1}$ (Viscozyme® L) from the original 1962 ± 10 kCal·g$^{-1}$. Dietary carbohydrates, including sucrose, glucose, and fructose have a caloric value of ≈3.9 kCal·g$^{-1}$. Non-digestible but fermentable carbohydrates, on the other hand, have a caloric value that ranges between 0 and 2.5

kCal·g$^{-1}$ (Roberfroid, 1999). FOS calorific value is estimated to be roughly 1.5 kCal·g$^{-1}$, which represents about 38 % of the energy value of digestible carbohydrates (Hosoya et al., 1988).

**Table 4.** Properties of the strawberry preparations before and after the enzymatic treatment with Pectinex Ultra® SP-L and Viscozyme® L.

| Parameter | Sample | | |
|---|---|---|---|
| | Non-treated | Treated with Pectinex® Ultra SP-L | Treated with Viscozyme® L |
| **pH** | 4.2 ± 0.0 | 4.7 ± 0.0 | 4.7 ± 0.0 |
| **°*Brix* (g·100 g$^{-1}$)** | 48.9 ± 0.1 | 48.5 ± 0.2 | 47.9 ± 0.1 |
| ***Aw*** | 0.940 ± 0.00 | 0.934 ± 0.00 | 0.936 ± 0.01 |
| **Relative Sweetness** | 514 ± 1 | 322 ± 2 | 305 ± 3 |
| **Caloric value (kCal·g$^{-1}$)** | 1962 ± 10 | 1490 ± 10 | 1600 ± 6 |

*aw*— water activity. Relative sweetness was calculated comparative to sucrose: fructose—173 %; glucose—74 %; sucrose—100 %;1-kestose—31 %; nystose—22 %; and 1$^{F}$-fructofuranosylnystose—16 % (Alasalvar et al., 2001; Yun, 1996).

In the EU, for a food product be considered energy-reduced, a reduction of at least 30 % in the energy value must be assured (Regulation (EC) No 1924/2006). The product developed using Pectinex® Ultra SP-L enzyme was able to reduce approximately 31 ± 1 % of the total energy concerning the original commercial strawberry sample. On the other hand, Viscozyme® L was capable of reducing the product caloric value by 26 ± 1 %. These results highlight the great potential of theses enzymes for *in situ* reduction of sucrose in food products.

The prebiotic preparations had approximately less 40 % sweetness than the non-treated ones. This outcome is consistent with information from the literature. FOS have less than one-third the sweetness of sucrose ($GF_2$: 0.31; $GF_3$: 0.22; and $GF_4$: 0.16) whereas glucose and fructose have 0.74 and 1.73, respectively (Alasalvar et al., 2001; Yun, 1996). Nonetheless, to overcome the lack of sweetness, artificial sweeteners like *aspartame* and *acesulfame k* can be considered. Moreover, the use of natural sweeteners such as *stevia* might be a good alternative to satisfy consumers' desire for healthier and *clean label* products. *Stevia* is a non-caloric sugar substitute, which is approximately 200−300 times sweeter than sucrose (Peteliuk et al., 2021). Therefore, only a small amount would be required to correct the loss of sweetness caused by the enzymatic-treated strawberry preparations.

Changes in physico-chemical characteristics of the strawberry preparations, namely, in pH, total solid content (TSS, *°Brix*), and water activity (*aw*) were also evaluated (**Table 4**). The TSS content was slightly reduced ($p < 0.05$) as compared to the non-treated sample. Since microbial spoilage is more likely to happen when sugar content is lower, the control over this parameter is of high importance. Data suggest that the decrease in *°Brix* was more significative in the sample treated with the Viscozyme® L. A higher percentage of FOS was synthesized by Viscozyme® L than that

of Pectinex® Ultra SP-L. FOS not being as soluble as sugars in water may explain the obtained outcome (Kumar et al., 2018).

Differences in the *aw* were also found as compared to the commercial strawberry preparation ($p < 0.001$). A lower *aw* will result in slower microbial growth, thus the reduction of this parameter is a positive outcome. FOS have a high moisture retaining capacity, which makes them excellent humectants (Ibrahim, 2021). The enzymatic strategies developed herein may help prevent spoilage and maintain the food product quality for longer storage periods. Lastly, the treated samples had a higher pH value than the non-treated sample ($p < 0.0001$). To enhance FOS synthesis, the strawberry samples' initial pH had to be adjusted to 5.0. After the enzymatic treatments the pH dropped to 4.7. Sodium citrate (E331), for instance, can be used to further lower the pH of the strawberry preparations to the manufacturer's required levels (4.0 ± 0.2).

### *3.6.2. Color changes in the strawberry preparations*

**Table 5** shows the evaluated color parameters before and after the treatments. All samples presented a reddish coloration, substantiated by the positive values of parameter *a**. Such behavior was expected given that strawberries are naturally red, and food colorants were added during the processing. Nonetheless, compared to the non-treated sample, the treated samples lost some redness (lower *a** values) and became more yellowish (lower *b** values). These results explain why the color slightly deviated (variations in $h^o$ value). The yellowish tint of the treated samples might be due to the enzyme's own yellow/brown appearance or as a result of the pigments' oxidation by temperature. Pigments may have been partially destroyed throughout the *in situ* process, and/or during the enzyme inactivation (90 ℃, 10 min).

**Table 5.** Color parameters of the strawberry samples before and after the enzymatic treatments with Pectinex® Ultra SP-L and Viscozyme® L.

| Color Parameter | Sample | | |
|---|---|---|---|
| | **Non-treated** | **Treated with Pectinex® Ultra SP-L** | **Treated with Viscozyme® L** |
| ***L**** | + 29.8 ± 0.1 | + 28.8 ± 0.02 | + 29.5 ± 0.1 |
| ***a**** | + 2.6 ± 0.1 | + 2.0 ± 0.0 | + 2.1 ± 0.0 |
| ***b**** | + 2.2 ± 0.1a | + 1.6 ± 0.0 | + 2.0 ± 0.1a |
| ***C**** | 3.4 ± 0.1 | 2.6 ± 0.0 | 2.9 ± 0.1 |
| $h^o$ | 0.87 ± 0.04a | 0.94 ± 0.03a | 0.71 ± 0.04 |

*L**— brightness(+) to darkness(−); *a**— redness(+) to greenness(−); *b**— yellowness(+) to blueness(−); *C**— chroma; and *h*°— hue angle. Analysis was performed in independent triplicates. Values are expressed in mean ± standard deviation. Values in the same row with a common letter indicate no statistical difference among treatments ($p \geq 0.05$).

A significant decrease in the luminosity ($L^*$) was also noted after the enzymatic treatments ($p < 0.05$), being more pronounced in the sample treated with Pectinex® Ultra SP-L. Apart from pigment oxidation, the heating during the course of the enzymatic treatment could have led to browning reactions resulting in a darker appearance. Cheng et al (2018) observed similar results, in which the lightness of longan juice was greatly decreased after the treatment with Viscozyme® L commercial enzyme. Note that, in their reactions, a temperature of 55 °C was used, which is slightly lower than the temperature used in this study (60 °C). Also, the presence of reducing sugars in the medium often results in *Maillard* reactions (Ayyappan et al., 2016; Emami et al., 2018) and could have been easily promoted during the enzyme inactivation step.

All the changes in the above parameters were reflected in a significantly less intense appearance (lower $C^*$ value, $p < 0.05$). The samples became less saturated, and the reddish color was less dominant. The saturation loss was more perceptive in the strawberry preparation treated with Pectinex® Ultra SP-L than with Viscozyme® L. Still, the changes in the appearance may not be noticed by the human eye as it is less sensitive than measurements using appropriate equipment. Herein, the total color difference ($\Delta E$) parameter was evaluated to test the difference between the treated and non-treated samples. According to the classification proposed by Cserhalmi et al (2006), the enzymatic treatments induced a slightly visible difference ($0.5 < \Delta E < 1.5$) in the strawberry preparations. These color variations were less noticeable in the samples treated with Viscozyme® L ($\Delta E = 0.7 \pm 0.1$), than in those treated with Pectinex® Ultra SP-L ($\Delta E = 1.2 \pm 0.1$).

Color plays a significant role in consumers' acceptability being one of the first attributes to be noticed. Pigments are highly susceptible to degradation during processing, often resulting in undesirable color changes in food products (Andrés-Bello et al., 2013). Strawberries, a key ingredient in the formulae of the studied preparations, naturally contain anthocyanins pigments. Anthocyanins are responsible for a wide array of colors, including various shades of pink, red, blue, and purple (Alappat & Alappat, 2020). This pigment stability is strongly influenced by temperature and pH, becoming more yellowish or colorless under unfavorable conditions.

### *3.6.3. Rheological and viscoelastic properties of the strawberry preparations*

Flow curves were obtained for both original (non-treated) and enzymatic-treated samples (**Fig. S2A, and S2B**). The experimental data were fitted to the Ostwald de Waele model, as it gave an accurate estimation ($R^2 \geq 0.99$) of the rheological properties. The consistency coefficient ($k$) and behavior index ($n$) of the strawberry preparation samples are presented in **Table 6**. The shear rate of 50 $s^{-1}$ was used as a reference to the oral phase (Vieira et al., 2021), and the respective apparent viscosity ($\eta_{ap,50}$) is also given.

**Table 6.** Rheological and viscoelastic properties of the prebiotic strawberry preparations before and after the enzymatic treatments with Pectinex® Ultra SP-L and Viscozyme® L.

| Rheological and viscoelastic properties | Sample | | |
|---|---|---|---|
| | Non-treated | Treated with Pectinex® Ultra SP-L | Treated with Viscozyme® L |
| $k$ (Pa·s$^n$) | 0.69 ± 0.01 | 0.11 ± 0.01a | 0.12 ± 0.01a |
| $n$ (Pa) | 0.63 ± 0.00 | 0.77 ± 0.00a | 0.76 ± 0.01a |
| $\eta_{ap,50}$ (Pa·s) | 0.16 ± 0.00 | 0.05 ± 0.00a | 0.05 ± 0.00a |
| $G'$ (Pa) | 4.23 ± 0.04 | 0.54 ± 0.07a | 0.49 ± 0.12a |
| $G''$ (Pa) | 4.09 ± 0.12 | 0.90 ± 0.09a | 0.83 ± 0.13a |
| tan$\delta$ | 0.97 ± 0.02 | 1.66 ± 0.05a | 1.74 ± 0.16a |
| $\eta^*_{50}$ (Pa·s) | 0.40 ± 0.01 | 0.10 ± 0.01a | 0.10 ± 0.02a |

$k$— consistency coefficient; $n$— flow behavior index; $\eta_{ap,50}$— apparent viscosity at 50 $s^{-1}$; $G'$— elastic modulus at 1 Hz; $G''$— viscous modulus at 1 Hz; $tan\delta$— loss tangent; $\eta^*_{50}$— complex viscosity at 50 rad·$s^{-1}$. Analysis was performed in independent triplicates. Values are expressed in mean ± standard deviation. Values in the same row with a common letter indicate no statistical difference among treatments ($p \geq 0.05$).

The enzymatic treatments highly reduced sucrose content, thus, changes in the behavior of the strawberry preparations were expected. All samples exhibit a non-Newtonian shear-thinning behavior, with the apparent viscosity decreasing as shear rate increases (**Fig. S2A, and S2B**). The preparations containing FOS and reduced sucrose content had lower consistency indexes ($p < 0.001$) as compared to the original product. The treatments caused a disruption in the gel network, and FOS were not capable of establishing a stable structure. Other authors have also determined a weakened structure for yogurts when adding FOS, as FOS interfered with the formation of the gel network (Pachekrepapol et al., 2021). The strawberry preparations also lost some pseudoplasticity and become more fluid, with the treated preparations presenting a higher $n$ and lower $\eta_{ap,50}$ compared to the original sample ($p < 0.01$). The lowest $k$ and $n$ values were observed for the samples treated with Pectinex® Ultra SP-L. Still, both parameters were not statistically different from that of Viscozyme® L ($p \geq 0.05$). The $k$ value was reduced approximately 6.3-fold and the $n$ increased by ~1.21-fold *prior* to the treatments.

The apparent viscosity of the enzymatic-treated products was reduced by 70 %. Roughly 50 % (w/w) of FOS and 32−35 % monosaccharides, *i.e.*, glucose and fructose, were present in the treated samples, with more than 80 % sucrose being converted. Both glucose and fructose have shown lower viscosity than sucrose (Chirife & Buera, 1997). FOS viscous character is correlated with the chain length (Verma et al., 2021). Short-chain fructans provide less consistency than long-chain fructans (Glibowski & Rybak, 2016). In the treated strawberry preparations only short-chain FOS (DP 3−5) were synthesized. Thus, a great loss in consistency was anticipated. Changes in the rheological behavior related to FOS supplementation and sucrose substitution has been reported in other food matrices. Greek yogurts with added FOS became less consistent and

viscous (Costa et al., 2019), and the partial substitution of sucrose with FOS resulted in lower dough consistency (Padma Ishwarya & Prabhasankar, 2013).

The influence of sucrose reduction and the presence of FOS on the samples network's properties were also evaluated (**Table 6)**. Changes in the strawberry preparations network's character were studied by analysis of the viscoelastic properties, *i.e*., elastic ($G'$) and viscous ($G''$) modulus, and respective loss tangent (tan$\delta$). $G'$ is related to the solid-like nature of the gel, whilst $G''$ reflects the liquid. The parameters were obtained from sweep oscillatory measurements. All strawberry samples exhibited an increase in $G'$ and $G''$ values with increasing frequency (**Fig. S3A**). Moreover, an increase in frequency led to a decrease in the complex viscosity ($\eta^*_{50}$) values (**Fig. S3B**), which is typical for shear-thinning behavior.

Up to a frequency of 1.3 Hz, the non-treated strawberry sample behaved more elastic rather than viscous ($G' > G''$, tan$\delta < 1$), exhibiting a more solid-like nature. From this point onwards, the sample showed comparable elastic and viscous character (tan$\delta \approx 1$). The results suggest that sucrose balance both behaviors and stabilizes the samples network at higher frequency levels. On the other hand, in the enzymatic-treated strawberry samples, the viscous character always outweighed the elastic one ($G'' > G'$, tan$\delta > 1$), indicating that the treated samples had a more a liquid nature. $G'$ and $G''$ were significantly reduced ($p < 0.001$) indicating a considerable loss of network's structure resistance to deformation. Also, compared to the original sample, the $\eta^*_{50}$ was reduced by 17 %. During the enzymatic treatment, the intermolecular interactions between the substances of the strawberry preparations must have been destroyed or weakened, resulting in a less structured network.

Rheological properties are of high importance in food processing as they greatly impact the product quality (Basu et al., 2017). Sucrose, the main sweetener used in the food industry, highly affects the rheological character of the food products (Torres et al., 2013). This sugar promotes hydrogen bonding between polymers and immobilizes free water, promoting gel formation and stabilizing the gel network (Muñoz-Almagro et al., 2021). For this reason, the manufacture of low-sugar products keeping comparable textural and rheological profiles is rather difficult.

## Conclusions

A prebiotic strawberry preparation was successfully produced by *in situ* enzymatic conversion of caloric sugars into functional ones. By applying the commercial enzyme mixtures Viscozyme® L and Pectinex® Ultra SP-L, a prebiotic strawberry preparation was obtained, with a content of more

than 50 % (w/w) FOS in total sugars, with less than 80 % of its original sucrose content, and with reduced caloric value (less 26−31 % calories).

The present study shows that over 2.5−3.0 g FOS (per 100 mL) would be ingested in dairy products containing 10 % of the developed prebiotic preparation. More than 90 % of FOS would reach the colon intact, clearly validating its prebiotic potential. The conversion of sucrose into FOS changed some physical and sensory attributes of the original product.

The developed *in situ* approach offers a unique strategy for the development of prebiotic food products and open up a new path for the production of low-sugar and low-calorie goods.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgments

This work was supported by the FCT under the scope of the strategic funding of UIDB/04469/2020 unit, by National Funds through the FCT under the project cLabel+ (POCI-01-0247-FEDER-046080) co-financed by Compete 2020, Lisbon 2020, Portugal 2020 and the European Union, through the European Regional Development Fund (ERDF) and by LABBELS – Associate Laboratory in Biotechnology, Bioengineering and Microelectromechanical Systems, LA/P/0029/2020. Daniela A. Gonçalves acknowledge the Portuguese Foundation for Science and Technology (FCT) for the PhD Grant (2022.11590.BD). The authors acknowledge Frulact SA for all support concerning the materials and information related to the industry sector.

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## Credit authorship contribution statement

**Daniela A. Gonçalves**: Investigation, Writing– Original draft preparation, Methodology, Formal Analysis, Visualization. **Vítor D. Alves**: Resources, Validation. **José A. Teixeira**: Project Administration, Resources, Supervision, Funding acquisition. **Clarisse Nobre**: Conceptualization, Methodology, Writing– Reviewing and Editing, Supervision. All authors have read and agreed to the published version of the manuscript.

# Article Highlights

- *In situ* enzyme treatments can be exploited to generate low-sugar functional foods.
- Fructo-oligosaccharides (FOS) were enzymatically synthesized at 60 ℃ and pH 5.0.
- A strawberry preparation with 50 % (w/w) FOS and less 80 % sucrose was obtained.
- The caloric value of the prebiotic-treated preparations was lowered in 26−31 %.
- More than 90 % of the FOS resisted to the *in vitro* digestion.

**Declaration of interests**

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Graphical abstract